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ART. IX.—*The Problem of Hard Seeds in Subterranean Clover.*

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Introduction.

It has long been known that a seed, though viable, may be unable to germinate under favourable conditions, through either dormancy of the embryo, or an impermeable seedcoat. A seed in which impermeability of the seedcoat prevents water absorption, is termed "hard", whereas a permeable seed is "soft". "Hardseededness" occurs characteristically in the Family Leguminosae and the hard seeds are capable of germination after any treatment which makes the testa pervious, such as cutting by contact with the rough sides of a threshing drum, scratching with sandpaper, slight charring with concentrated sulphuric acid or hot ashes, contact with boiling water. Softening by means of mechanical treatment is important in the commercial harvesting of leguminous crops such as clovers and lucerne. Natural softening in field and store is of significance in the practical value of the species concerned.

The literature on hardseededness has been reviewed by Witte (10). Since 1932, Helgeson (6), Hamly (4), Stevenson (9), and Dutt (3) have contributed valuable information. However, a thorough investigation is still needed over the whole problem of hard seeds in Legumes, in order to understand the sequence of events from the production of a hard seed to its ultimate softening.

Subterranean clover was selected for study on this plan, as a contribution towards such a survey; not only was its formation of hard seeds of economic significance, in its position of the most important annual legume in Australia, but it had the unique characteristic among clovers of burying its seed, and the relation of seed-burial to hardseededness was not known.

The habit of the Subterranean clover plant, its type of flowering, the tendency to bury the flower cluster after fertilization, and the formation of a burr round each group of three or four seeds, are shown in plate XI.

Observations and experiments were made on all available material, including burr of Mt. Barker variety from a commercial grower, commercial samples of seed, and hand-cleaned seed from the strains grown at Burnley Gardens. These were grouped as Early maturing—Dwalganup, Mulwala, Dalliak, Springhurst, and Bacchus Marsh; Midseason—Mt. Barker, White Seed, Mansfield, Nangeela, and Burnerang; and Late maturing—Berlin, Merino, Macarthur, Tallarook, Wenigup, and Bass.

The extent of hardseededness in this clover is indicated by two facts, (1) that each burr usually contains both soft and hard seeds; (2) that in 1937, the percentage of hard seeds varied from 50 per cent. to 95 per cent. in the numerous samples examined less than a month after the burrs of the latest strains had dried—results similar to those of Meadley (7), in the district of Boyup, Western Australia.

Formation of Hard Seeds.

(1) *Relation of Hardness to Seed Development.*

In a normal spring, about one month elapses between fertilization and the growth of the young seed to its maximum size. The seed then dries, and becomes mature.

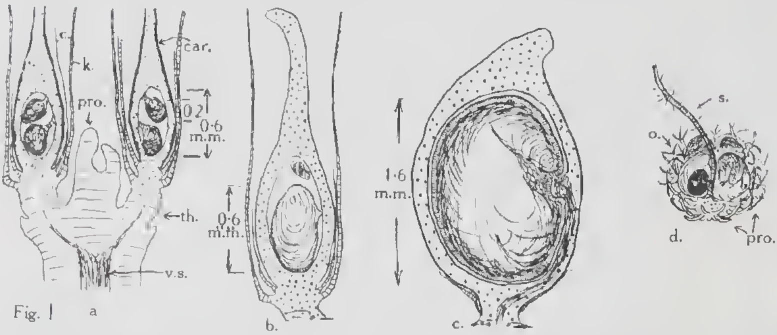


FIG. 1.—Longitudinal sections of the developing ovule of *Trifolium subterraneum;* (*a*) through the florets in a panicle at fertilization, showing two ovules in each ovary; (*b*) through an ovary some days after, showing growth of one ovule only; (*c*) a seed several weeks old, half grown; (*d*) diagram of a mature burr, showing the rosette of infertile calyces round the pods, and a pod opened to show the dried seed within. (v.s. = vascular strand, th. = thalamus, k. = calyx, pro. = primary infertile flowers, c. = petals, car. = carpel, o. — pod, with dried seed exposed, s. = stalk of burr.)

The ovary at fertilization is about 1 mm. long and usually encloses two ovules each about 0.2 mm. long and 0.15 mm. wide (fig. 1*a*). As a rule only one of these ovules develops in each ovary (figs. 1*b*-*d*). When the seed is about 2 mm. long, it is tightly enclosed by the greenish white membraneous ovary wall.

At this stage the testa is colourless. A faint pink soon appears round the hilum and spreads over the seed. The full-sized seed is dark crimson except at the strophiolar and micropylar regions which are paler, and at the hilum which is white owing to the disc of loose cells connecting it to the vascular strand of the ovary. The normal process of drying results in a reduction in the diameter of the seed from about 4 mm. to about 2 mm. and a change in its colour from crimson to purple-black except round the hilum which remains somewhat lighter.

Changes in the permeability of the developing seed were tested by using 1 per cent. osmic acid. The testas of young seeds were rapidly permeated by the acid over most of their surfaces. As development proceeded, the permeable areas diminished in extent. In seeds at their maximum size, the staining occurred as patches scattered over the surface (fig. 8*a*). From this it may be assumed that entry of water is possible over most of the testa before the seed dries. If staining occurs at the strophiole, it is a thin line across the centre. After the drying, the matured seed remains permeable, forming an "original" soft seed, or becomes hard, and this hardness is of variable duration. The seeds that soften, gradually raise the total soft seed percentage in the burrs despite the later hardening of some of the original soft seed. Osmic acid stains the soft seeds derived from hard, with a black spot at the end of the seed nearer the hilum (fig. 8*c*), while the original soft seeds are stained at the radicle tip, or in nearby patches (fig. 8*b*).

(2) *Occurrence of Hardness in the Individual Plant.*

It was noticed that, in many panicles, all the florets did not open at the same time, and this was followed by uneven development of the seeds, as evidenced by differing sizes, and by the variation in the time at which they began to colour pink. It was probable that this unevenness was connected with nutritional retardation in the less advanced seeds, and that these seeds would tend to remain soft, or develop a hardness of short duration. To test the idea, cotton was tied round the base of retarded florets, and the seed of each was separated from the rest in the burr, after ripening and drying out. There was an indication that least advanced florets in the panicle gave rise to some of the seed that softened first.

The position of the burr above or below the soil surface affects the size and time of drying of the seeds. Among burrs of the same age, those developed above ground are green and half the size of the buried burrs which are white. They dry out much later than those on the surface, because of the lowered maximum temperatures (Table 1) and more moist conditions. The seeds in the buried burrs are slightly larger, and the number of seeds matured per burr more than in the surface burrs; the increase seems due to the longer time for development before

TABLE 1.

Daily maximum and minimum temperatures in degrees Fahrenheit for air, soil surface, and just below surface, November and December, 1938.

Date.	Air.	Soil Surface.	Date.	½-in. below Surface.	Soil Surface.
Nov. 25 ..	52–74	50–103	Dec. 3 ..	50–116	47–125
,, 26 ..	48–82	46–106	,, 7 ..	56–94	52–100
,, 28 ..	60–94	56–108	,, 19 ..	48–112	46–123
,, 29 ..	52–82	46–108	,, 22 ..	40–110	46–122
			,, 28 ..	–112	–131
Average ..	53–83	49–106			
Variation in Max. Temp., 20° F.			Variation in Max. Temp. 10–20° F.		

drying. That length of development is linked with hardseededness was shown in an experiment with a row of plants of Bacchus Marsh strain. Burrs on one side of the row were encouraged to develop below the soil surface, which was kept moist till about a month after the drying of the burrs on the other side, where they had been prevented from burying. Seeds from the buried burrs, after drying on the plants, proved to be slightly more hardseeded than the surface seeds. Further drying of both types under a very low saturation deficit, caused an increased percentage of hard seeds in the buried burrs, which were evidently of higher potential hardseededness than those formed above ground.

The evidence that the stage of development of the seed at drying was important in determining its subsequent hardness, is supported by the distribution of hard and soft seeds according to the position of burrs along a runner. In plants or burrs collected while green, and allowed to dry, seeds from the younger burrs contained a higher percentage of soft seed than from the older—as shown in Table 2. Burrs from a plant of Dalliak strain were among those analysed in greater detail, and shows the same trend in Table 3.

TABLE 2.

Variation in yield of soft seeds along the runner. Burrs from 1937 harvest, Burnley, tested 10th January, 1938; plants cut in November while green. (Approx. 200 seeds per strain.)

Strain.	Percentage of Soft Seeds in Burrs.	
	Basal (First to Third Burrs).	Upper (Sixth Burr and Higher).
Berlin	14	52
Tallarook	1	15
Madrid	16, 25	42, 40
Dalliak	1	14

TABLE 3.

Typical analysis of a plant harvested green. The occurrence of softness according to burr position is stated.

Position of Burr along Runner.	Soft Seeds.	Total Seeds.	Percentage of Soft Seeds
			%
Basal 1	18	76	28
2	19	56	
3	17	47	
Mid 4	19	68	
5	9	31	33
6	21	58	
Upper 7	10	40	
8	14	27	40
9	5	22	
10	5	18	
11	7	12	70
12	7	9	

It would be expected from this, that in a range of strains grown together, the advent of hot, dry weather towards the end of the growing season would tend to dry off the youngest seeds of the later stains before they were fully developed. This would cause a characteristically higher percentage of soft seeds than would occur in earlier strains in which the seed had longer time to

TABLE 4.

Variation in soft seed yield a/c strain and position of flower along runner. (Seeds collected from Burnley plots in mid-November and tested at end of month.)

Strain Maturity.	Name.	Soft Seeds.	Total Seeds.	Percentage Soft a/c Position.
				%
Earliest ..	Dwalganup ..	3	51	Basal .. 0
				Mid .. 6
				Upper .. 20
Early	Mulwala ..	15	43	B. .. 16
				M. .. 65
				U. .. 60
Early-mid ..	Springhurst ..	17	83	B. .. 7
				M. .. 25
				U. .. 41
,, ..	Bacchus Marsh	32	117	B. .. 11
				U. .. 42
Mid-season ..	Mt. Barker ..	6	26	B. .. 22
,, ..	White Seed ..	10	41	B. .. 18
				U. .. 57
,, ..	Mansfield ..	35	64	B. .. 55
,, ..	Nangeela ..	22	56	B. .. 40
,, ..	Burnerang ..	46	48	B. .. 96
Late	Merino ..	31	37	B. .. 84
,, ...	Macarthur ..	36	39	B. .. 93
,, ...	Tallarook ..	16	16	B. .. 100

develop. This seems to be borne out by field observations up to date. A late strain (Tallarook), had a relatively high percentage of soft seed, in December 1937, and a heavy germination in the ground after the rains in January 1938. The early strains (Dwalganup, Dalliak, and Mulwala) had few soft seeds, and a very low germination in the plots. The general correlation of softness with lateness of maturity is seen in Table 4, but the variation in results indicates the necessity for further data on the effect of variety on hardseededness, as distinct from its effect on the time of seed development.

The degree of drying of the seed was found to be important in hard seed formation, when burr from the same varieties was gathered at intervals from October to December 1938. Each set was further dried under standard conditions, and then tested. The longer the seeds were allowed to dry out on the plant, the higher the percentage of hard seed (Table 5).

TABLE 5.

Variation in soft seed yield a/c strain and time of harvesting.

Strain.	Percentage Soft Seeds.—Time of Harvesting, Nov.–Dec., 1938.		
	15/11.	23/11.	14/12.
	%	%	%
Bacchus Marsh	40	..	30
Mt. Barker	72	40	..
White Seed	80	..	23
Macarthur	100	53	..
Tallarook	100	51	18

Observations were made on the variation in hardseededness in the plants of a strain row. In each of several strains, twelve individuals were selected out of about 90, and the burrs dried further under standard conditions before testing in December. There was a wide range, due to a few plants having soft seed percentages well away from the mean.

Strains are chiefly distinguished by their differential response to environmental conditions, particularly as to time of flowering. A previous paragraph has indicated the correlation of late strains with less hardseededness, because of the less development of seeds before drying. A further confirmation of the importance of seed development was obtained by causing a number of strains to begin flowering several weeks earlier than usual for the Melbourne district. Earlier flowering resulted from growing the strains at Swan Hill, Cohuna, and Rutherglen. Sufficient watering enabled the flowering plants in the northern districts to grow until December, and so lengthened the growing period by about three weeks. The flowering period was thus increased by about a month. This resulted in all strains being more hard-seeded than those at Burnley, when sampled at the end of November. The hard seed percentage in plants of the nine strains, grown at Swan Hill and Burnley, are compared in Table 6.

TABLE 6.

Variation in soft seed yield a/c strain and district. Harvested end October, 1938.

Strain.	Percentage Soft Seeds.	
	Melbourne.	Swan Hill.
Dwalganup	Basal .. 47	4
	Upper .. 60	42
Mulwala	B. .. 60	17
	U. .. 70	75
Springhurst	62	B. .. 6
		U. .. 36
Bacchus Marsh	B. .. 12	12
	U. .. 60	12
Mt. Barker	72	B. .. 36
		U. .. 41
Nangeela	65	20
Burnerang	..	30
Macarthur	100	35
Tallarook	100	92

(3) *Factors Influencing Formation of Hard Seeds.*

From the foregoing observations on the occurrence of hard seeds in the plant, and in the strain, it is indicated that (1) length of development of the seed determines its capacity to become hard; (2) degree of drying influences its attainment of hardness. Experimental evidence on the importance of dehydration is summarised below.

Several workers, notably Helgeson (6) and Dutt and Thakurta (3) have noted the effect of degree of dehydration on the formation of hard seed. In both *Melilotus alba* and *Cajanus indica* it was found that where seeds which had reached maximum size were subjected to a certain amount of drying, germination could invariably ensue under favourable conditions, but on further drying, impermeability developed, which however could be reversed at once, by scarifying.

The effect of dehydration on full-sized seeds was therefore investigated, using different environments, and different times of exposure to one environment. Burrs were obtained from plants of the Dwalganup strain, which had been sown in December and had begun flowering in March. The seeds were 4 mm. long and the testas deep red. Four quadruplicate samples of 25 were used in each of the tests in Table 7; the test begun 30th June, 1938, varied the rate of dehydration in one time; and the test begun 14th July, 1938, varied the time, the rate being constant.

TABLE 7.

Effect of dehydration on hard seed formation.

Date Commenced.	Length of Time.	Temp. Deg. Cent.	Relative Humidity.	Saturation Deficit.	Percentage Hard Seed.
			%		
30·6·38 ..	12 days ..	15	80	0·11	0
		15	0	0·54	40
		29	35	0·93	65
		29	0	1·41	75
14·7·38 ..	18 hours ..	29	0	1·41	15
	42 hours ..	29	0	1·41	17
	72 hours ..	29	0	1·41	35
	11 days ..	29	0	1·41	52

It is evident that in *Trifolium subterraneum*, the degree of dehydration controls the formation of hard seeds from previously permeable full-sized seeds. The factors in this dehydration are temperature, relative humidity, and the length of time during which they can act. The drying effect of high temperature and low relative humidity is expressed more briefly in terms of saturation deficit. It may therefore be said that the more intense the factors of saturation deficit and of time, the more hard seeds are formed in the burrs of any sufficiently mature plant.

(4) *Comparison of Hard and Soft Seeds.*

Storage in moist air will cause an obvious absorption of water on the soft type, but not on the hard which is unable to absorb water into the seed below the testa. After such a process, the hard seed will be black all over or at most, slightly pink at the hilum; the soft seed will be slightly larger, and purplish black except the area which includes the hilum, which will be distinctly pink.

Hard and soft seeds can be separated by this colour difference in samples of burr harvested later than usual in autumn after exposure to weather conditions of varying moisture since their ripening in December. This difference can also be readily observed in commercial seed stored several years under a low saturation deficit. In freshly dried burrs, however, the two types of seed are indistinguishable.

In order to determine the absorption of water vapour by hard and soft seed, samples of 200 seeds of both hard and soft types (distinguished by swelling capacity) were brought to constant weight in a desiccator and then exposed to room conditions

(70–80% R.H. and 16° C. + 2) and weighed periodically. Fig. 2 shows the results. In 30 days, the percentage increase of weight in the soft seeds was three times that in the hard. This effect is reversible.

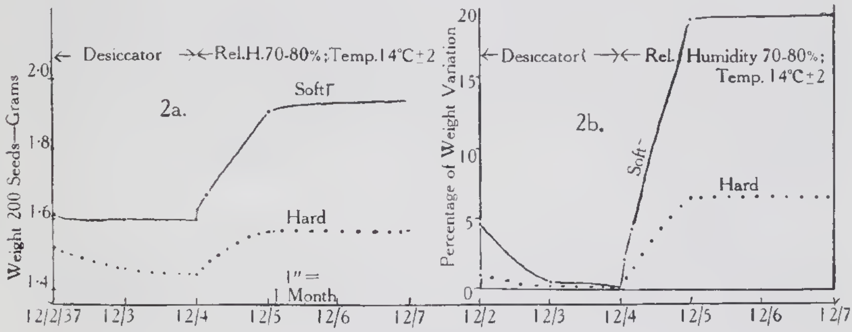


Fig. 2.—Absorption of water-vapour by hard and soft seeds; (*a*) showing relative weight changes; (*b*) showing changes in weight as a percentage of weight of each type, after two months in desiccator.

Hard seeds are evidently able to absorb some water vapour, and other tests show that water soluble dyes penetrated the matrix of hard seeds but no further, whereas they stained the whole testa in soft seeds. Since the anthocyan colour in the testa is situated below the impermeable layer, the addition of water to hard seeds might be expected to have no effect on colour, the matrix only, being affected. Conversely, the penetration of moisture in the soft seed lightens the testa colour and causes the characteristic pink appearance of the hilar region.

Soft seeds should generally be larger than hard because of their greater capacity to absorb water vapour. This was proved in a test on two-years old samples of seed, from burr collected in the field and from a commercial source. They were graded on a 2 mm. square mesh sieve, and those that passed through were put through a 2 mm. round mesh sieve. The percentages of soft seed in each grade are shown in Table 8.

Table 8.

Effect of seed size on soft seed percentage. (Samples of 200 seeds, each duplicated.)

Graded 2 mm. Square Mesh.	Percentage Soft.	
	Burr.	Commercial Seed.
	%	%
Graded > 2 mm. square mesh	35	94
,, < 2 mm. square mesh	14	88
,, < 2 mm. round mesh	5	79

The effect of the higher water content of the soft seed is evident in the soft seeds being about 15% heavier than the hard under ordinary storage conditions.

On a dry weight basis, however, soft seeds are about 20% lighter than hard in the same sample. Samples of 100 seeds of both hard and soft types, from single plants and bulk sources, were weighed, dried at 100° C. for four days, and weighed again. Taking one result, 100 hard seeds weighing 0.6535 grams, were dried to 0.6228 grams after a loss of 4.7% water; 100 soft seeds weighing 0.7123 grams were reduced to 0.4898 grams, after a loss of 30% water.

The higher dry matter content of the hard seed might be expected from the correlation of length of seed development with capacity for hardseededness. A long period of development should mean a high nutrient concentration and hence a high dry matter content, and the evidence supports this assumption.

The Softening of Hard Seeds, under Natural and Commercial Conditions.

The natural causes for the softening of hard seed in storage or soil are of interest to any concerned with the agricultural value of such seed. A review of the literature showed only isolated tests on single factors thought to be of importance—namely, temperature, mechanical pressure, humidity, alternation of wet and dry conditions.

Information was obtained on the occurrence of hard and soft seed in burrs harvested in March, 1936, by Mr. W. W. England, of Warncoort, Victoria, and received in May. Most of the burrs contained three or four seeds and separation of the results into those two classes showed that the percentage of soft seed in each did not differ significantly from a total of 20 per cent.

A later count of burrs containing 5, 4, 3, and 2 seeds gave similar results. There is no evidence to suggest that the occurrence of soft seed is influenced by the total number of seeds per burr. The staining of these seeds with osmic acid showed that most of the seeds which were soft, had been hard. The softening must have taken place between early January and May.

Under field conditions, burrs gave a certain percentage of soft seed at first, followed by decreasing amounts during the next months and years.

Conditions of storage affected the rate of softening in these burrs. The percentage of soft seed in burrs kept both dry, and wet occasionally, in a well-ventilated concrete building subject to daily fluctuations of 20–30°F., was significantly greater than in the rest of the burr stored in the soil laboratory with a daily range of 2–5°F. Fig. 3 records that in the latter, even after two years, there is no significant increase from the original 20 per cent., whereas, in the former, the soft seed percentage has increased to 50 per cent. Similar burrs tested for four months under the range of conditions in Table 9 showed similar increased softening with wide daily temperature ranges.

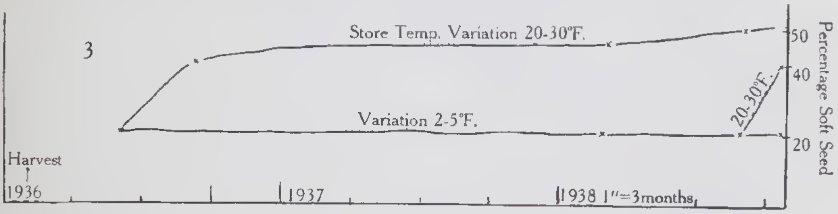


FIG. 3.—Rate of softening of seeds in burr, as affected by high and low daily ranges of temperatures.

TABLE 9.

Place.	Range of Temperatures.	Percentage of Soft Seeds after Period Aug. to Nov., 1938.
		%
Bulk store of 1938 burr ..	4° F.	18 (no increase on original test)
Oven at 29° C.	4° F.	26
Window ledge	60–110° F.	34 (distinct increase)
Buried in soil in plot ..	40–120° F.	50 (distinct increase)

From the above observations, the importance of environment on softening in soil and storage is apparent. The possible factors of this environment are temperature, humidity, soil microflora that might assist in breaking down the outer part of the seedcoat, and alternate wetting and drying.

In order to test, under conditions of favourable culture and at a constant temperature of 28°C., the effect of the main types of microflora which may occur on and around the seedcoat of hard seeds, the following experiment was carried out. Moulds were favoured on a medium of glucose agar, root nodule bacteria on yeast mannite and other possible testa saprophytes on a silica gel. Four intensities of infection were used, viz., sterilized seeds, ordinary seeds, seeds with burr debris, and seeds with soil. About 200 seeds from the Warncoort burrs were used in each dish so that the first to germinate were those already soft.

In examining the results after four months and a year's treatment in such media, the first germination (about 20 per cent.) was ignored except to find the variation and only the later germinations analysed for significance. It was found that variation between both the different types and intensities of infection was not significant after these periods.

Fig. 4, of the amount of softening in time in the three media shows how gradual the softening is. The sudden rise in the last week in May occurred after an accidental rise in temperature to 31° and then a fall to room temperature (10–15°C.) in a week-end. Otherwise, the temperature remained at 28°C. constant throughout the test. On 28th June, 1938, the seeds were transferred to fresh media, but no marked rise followed, despite the vigorous growth of fungi and baeteria colonies.

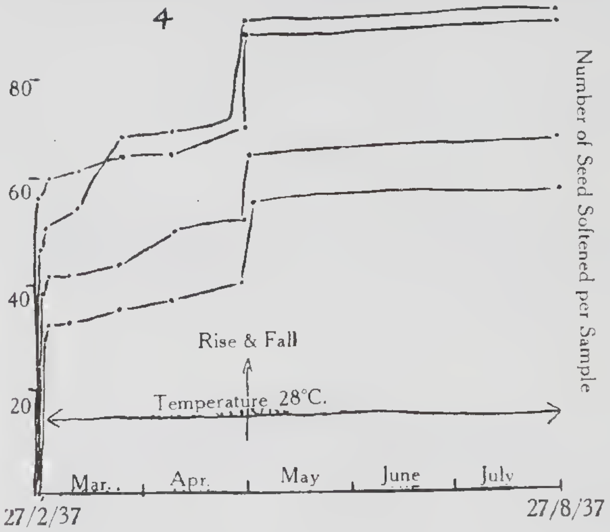


FIG. 4.—Rate of seed softening in Experiment 1 (effect of soil microflora), showing the effect of a wide temperature fluctuation.

Two strains of fat-splitting bacteria were tested on hard seeds in a broth medium, with negative results. Certain moulds and bacteria may become important after a longer period than a year but since much softening occurs within the year, the reason for this must lie with the remaining factors—temperature and moisture.

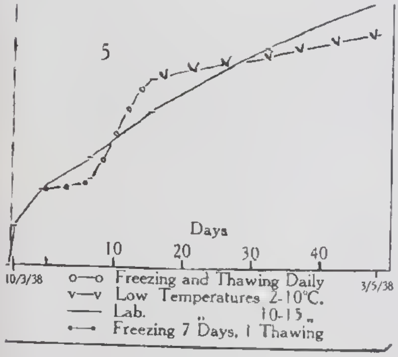
A second experiment was planned to include constant high and low temperatures and varying temperatures, with seeds continuously wet, and alternately wet and dry. In addition, seeds were tested in water changed daily, and in stagnant water contaminated with fragments of burr and hulls, and the germination was noted in burrs sown in soil in an outside plot, under normal conditions of fluctuating temperatures, moisture and microflora.

The standard error was the same as in the previous experiment, and hence the results of softening to be significant had to be more than 15 per cent. greater than the controls. Results from the 10th February to 8th April, 1937, show none significantly greater except possibly one which had been placed so as to get direct sun, and the widest temperature range possible in the laboratory (15°–40°C.). After the 8th April, it was moved out of sunshine and the softening rate became the same as most of the others.

At the end of the testing period, 8th March, 1938, the tests with two exceptions had softened about 47 per cent. The two exceptions (with 60 per cent. soft seed) had experienced the same accidental rise and fall in temperature as in the previous experiment.

These two experiments indicate that the fluctuation of temperatures is an important factor in inducing softening.

A third experiment was carried out to test the effect of freezing for seven days compared with freezing and thawing every day for seven days; and the effect of similar temperature range but not including freezing, with controls at laboratory temperature. Hard seeds of three successive harvests were used in each case, and the results showed decrease in sensitivity to temperature ranges above freezing point with increase of age. One long freezing softened the younger seeds more than the older, but a week of daily freezing and thawing to 15°C. softened a third of all the hard seeds irrespective of age. Fig. 5 shows the effect of temperature range on seed from 1937 burrs. Softening increases gradually, under 10°–15°C., while freezing over some days, or keeping at a range of 2°–10°C. retards the rate, and alternate freezing and thawing increases it conspicuously. Seed harvested in 1936 and 1935 reacted strongly to the softening effect of alternating freezing and thawing but, in contrast to 1937 seed, it was not softened at room temperatures (fig. 6).



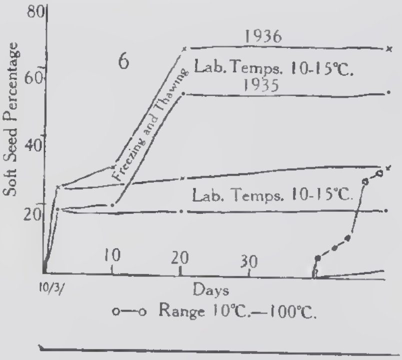


FIG. 5.—Rate of softening of seeds from 1937 burr, under various temperature ranges.
FIG. 6.—Comparison of rate of softening of seeds from burrs of 1935 and 1936 harvest, under conditions of freezing and thawing daily, for a week.

A fourth experiment was planned in order to try the effect of a wide temperature range on these less sensitive seeds. Such seeds softened considerably when subjected to such a high temperature of short duration as caused by contact with boiling water in which they are let cool. The results of repeated alternation of 10°–100° C. gave as much softening as the repeated freezing and thawing, viz. (fig. 6). The effect of a boiling water application at night was evidenced in swollen seed the next morning.

The same result was found, though to a much less degree, with seed kept in the 28° C. incubator during the day and taken out into room temperature (10° C.) at night, as compared with no softening in seed kept constantly at 28° C.

The special effect of freezing and thawing, out of proportion to its temperature range; the increased influence of a given temperature range, the higher its mean level; and the increased softening with wider ranges and lack at constant temperatures; the modifying effect of age of hard seed on sensitivity, have all been noted experimentally. They point to the major role played by the temperature factor in the softening of hard seed under natural conditions.

It is well known that the high percentage of hard seeds usual in a burr sample of *Trifolium subterraneum* is very much reduced by the cleaning process in commercial seed production. Meadley (7) states that a variation of 14–45% soft seed in hand-cleaned samples from one small district was observed, whereas from machine-dressed lots, there was a range of 44–96%. This increase was entirely due to efficiency of the machine and the weather conditions at threshing. Hamly (4) discovered that the softening was due to the bouncing rather than to the scarifying action involved, and demonstrated in *Melilotus alba* and some clovers, the importance of an area in the seed more sensitive to stresses than elsewhere. Any break in the testa could be seen by a black stain developed with osmic acid. In the light of this knowledge, hard seeds (water tested) of subterranean clover were examined, and found to soften under the same method of shaking, after which treatment the softened ones showed the same type of staining with osmic acid, as recorded by Hamly.

Table 10 summarizes the effect of the osmic acid test on seeds after differing treatment. It confirms Hamly's results that (1) hard seeds, and soft seeds formed from hard, differ only in permeability of testa through splitting of outer cells, or by scarring by mechanical scratching. (2) That hard can be changed to soft by shaking. (3) That the area most susceptible to splitting is a special one—the strophiole. (4) That commercial seeds show far more soft seed through strophiole splitting than through external damage to the testa or because of original discontinuity of the impermeable layer round the testa. Table 11 shows the effect on softening of several mechanical methods of testa treatment, and the efficiency of the shaking or "bouncing" method.

Hence it is seen that the large increase in soft seeds found after the commercial threshing of subterranean clover burr is due to the bouncing action in the drum.

TABLE 10.
Identification of soft seed by Hamly's Osmic Acid method.

Source of Seed.	Result after five minutes in 1% Osmic Acid aq. soln.
1. Burr	Several with black spot at strophiole, and several with no marking
2. Commercial seed	Majority with black dot at strophiole, and few others with black scars elsewhere
3. Hard seed after 40 mins. conc. sulphuric acid	Black scars over testa
4. Hard seed shaken 100 times in bottle	As for (1)
5. White seed from burr ..	As for (1)

N.B.—The seeds that showed any black spot proved to be soft seed when put in water long enough to allow sufficient imbibition for swelling.

TABLE 11.
Effect of methods of Testa Abrasion on seed softening. (Duplicates of 25 burrs and 100 seeds.)

Treatment.	Per Cent. Soft Seed.
	%
1. Commercial seed	84
2. Seed from burr	20
3. Dehulled from burr	13
4. Dehulled from burr after conc. sulphuric acid for 40 minutes, but unstirred	18
5. Same, but stirred	52
6. Seed shaken 600 times in bottle	91

From the preceding information, in Sections II. and III., come the following facts:—(1) The importance of dehydration in hard seed formation; (2) prominence of fluctuating temperatures in softening hard seeds under field and storage conditions; (3) the influence of "bouncing" seed, on a special area—strophiole—during the preparation of commercial samples, in reducing the normally high hard seed content. A close examination of the seed in development and after maturity showed the internal reasons for these facts.

Relation of Seed Structure to Hardness.

So far it has been shown merely that hulled seeds are permeable to water before drying out, after which they may or may not be. Sections were taken to see the connection of the testa structure with the development of impermeability. Fig. *7a* shows the situation of the testa in a pod near full size. It was found that in later stages of the seed, the outer (Malpighian) cells went through a series of changes. At first they were colourless, and their distal ends were convex. Later a slight subcuticular layer (matrix) formed and the surface of the cell tips became flattened, then pink colour developed all through the cells. Withdrawal of the colour from the tops of the cells preceded the formation of a thicker matrix and a thin cuticle. The matrix and decolourized cell tips appear as a lighter area above the darker cell walls beneath, without any definite translucent line at the junction. These changes in the testa, and also the accompanying crushing of the nutrient layer, and the lessening of the cell cavities after drying out, are shown in fig. 7 (*b-d*).

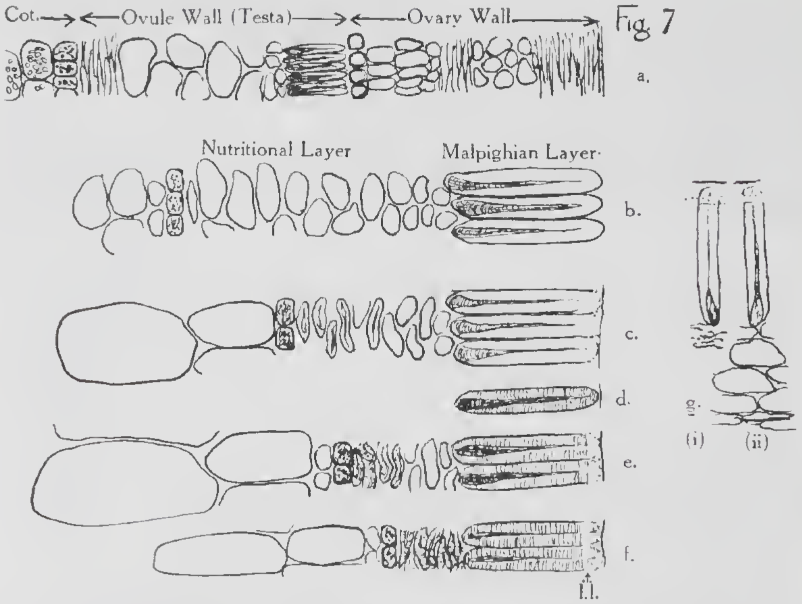
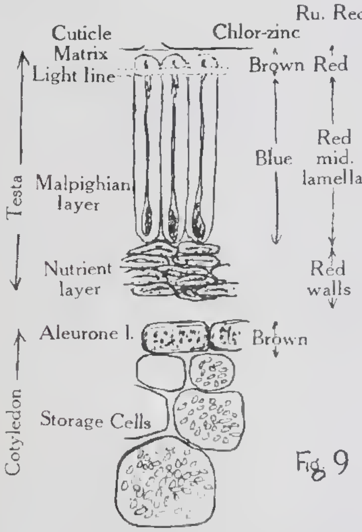


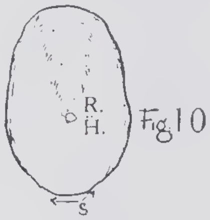
Fig. 7.—Longitudinal sections through the testa, showing (*a*) its position in the pod; (*b-f*) the changes in its cells from before the seed colours till it becomes hard; (*b*) Malpighian cells with rounded tips; (*c*) tips flattened and cuticle formed; (*d*) colour throughout cell; (*e*) colour withdrawn from tips; (*f*) reduction of cell lumens and formation of lightline, after drying of seed; (*g*) increase in depth of nutrient layer after contact with water.

A closer study of the time of development of the light line was made using well developed seeds and varying intensities of drying. Chlorzinc iodine and safranin were used as stains to differentiate cellulose from altered substances and hand sections were made of fresh seeds. Close microscopic examination showed that at the stage when the seed was beginning to colour externally, the Malpighian cells gave an even cellulose reaction (Plate XII., A). At the stage of full development, the stain was lighter at the tips. The same staining occurred in seeds that had been dried four days but were still soft. However, in hard seeds resulting from this treatment, there was a colourless band at the top with a light line more or less in evidence at its junction with the coloured lower cells. Soft or hard seeds from commercial samples showed the same type but with the light line more conspicuous, and the matrix yellow. (Plate XII., C.)

The strophiole must be described apart from the rest of the testa for it was found that the light line appears there before the seed is fully formed. This seems linked with an earlier development of the Malpighian cells. In seeds about 1 mm. long, the strophiole cells were three times the length of the ordinary testa cells, more vacuolated, and had less conspicuous nuclei. At 2 mm. a more or less distinct light line was evident between the lower cell walls and a definite matrix (Plate XII., B). At this stage all the testa except this area was readily permeable. It was concluded that the presence of the light line indicated that of a substance impermeable to water, and though the light line was apparent at the strophiole before dehydration of the seed, its presence throughout was necessary for impermeability.

In mature seeds both hard and soft, the testa consists of two distinct layers of cells (fig. 9). The Malpighian layer is the deeper of the two, its cells being usually 3μ in diameter, and 24μ long. The outer surface of these is covered by a thin cuticle over a substance $1–2\mu$ thick which reacts to pectin dyes but does not swell on contact with water. That water can penetrate the cuticle and matrix is shown by the staining of these by such water soluble dyes as Methylene and Nile Blues and Crystal Violet.





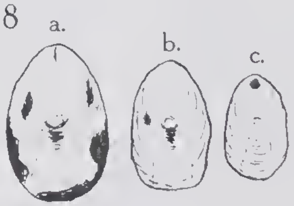


FIG. 8.—Staining reaction of seeds with osmic acid 1 per cent. for 1 minute; (*a*) at maximum size, there are scattered patches, and always at radicle tip; (*b*) dried seeds, still soft usually stain at radicle tip; (*c*) soft derived from hard develop circular stain at strophiole.

FIG. 9.—Diagram of the testa of a mature seed, with the colour reactions of some chemical tests.

FIG. 10.—Ventral view of a seed, showing hilum (*H*), radicle (*R*), and strophiole (*S*).

The walls of the upper ends of the Malpighian cells (termed "caps" by Hamly) are thickened by a material impermeable to water. This property is due to suberisation, as indicated by the cerinic acid reaction and other tests summarised in the following table, in which names at the head of columns refer to the layers in fig. 9.

TABLE 12.
Reactions of Testa.

Test.	Cuticle.	Matrix.	Suberized Layer.	Cellulose Walls.	Pectins of Nutrient Layer.
Phloroglucin and HCl. (Lignin)	..	..	..	Pink (·.·anthocyan)	
Cerinic acid (Suberin)	..	..	Yellow fat drops		
Chromic acid (Suberin)	..	..	The most resistant	Brown	
Crystal Violet (Fats)	Blue	Light violet	..	Lighter violet	
Schweitzers Reagent (Cellulose)	..	Swollen granular	..	Blue ..	Clear
Conc. H_2SO_4 and I. (Cellulose)	Dark	Dark ..	..	Mauve	Bluish to yellow walls
Chlor-zinc iodine (Cellulose)	..	..	..	Blue ..	Clear
HCl. then Amm. 2 per cent. (dissolves pectins)	..	Dissolves			
Ruthenium Red (Pectins)	..	Pink ..	..	Pink, mid lamellae	Pink
Methylene Blue (Pectins)	Violet	Bluish ..	..	Greenish	Blue

The junction between the suberised area and the unsuberised walls below is distinguishable as a translucent refractive line about 1μ thick occurring 3μ below the cuticle, and known as the "light line." The cause of this line has been shown by Hamly (4), to be due to the contact of substances of differing refractive indices.

Below the light line the walls of the Malpighian cells are of a cellulose nature, as seen from their reaction with chlorzinc iodine and sulphuric acid, but contain also certain substances that cause some reduction of osmic acid and a consequent black stain. The lumen of the cell decreases in width in the upper two-thirds of its length till it becomes about 0.3μ. Staining with osmic acid shows that several processes project vertically in the lumen just below the suberisation. In the dry seed, the cell contents are confined to a small deposit at the base of the cell, and noticeable only when stained by such as crystal violet or osmic acid. No chromatophores are visible as are characteristic of the Malpighian cells of *Albizzia*.

The inner layer of the testa consists of about five rows of dead, collapsed cells of the "nutrient layer." These have a great capacity for swelling in the presence of water, due to the high proportion of pectin in their walls, e.f. fig. 7 *g*. In *T. subterraneum,* the osteosclerid layer, occurring between nutrient and Malpighian layers, and typically formed of hourglass cells, is not sufficiently distinct to be separated from the nutrient layer. This is an exception to the general rule established by Pammell (8) for most leguminous genera including Trifolium.

The outer tissue of the cotyledon occasionally adheres to the testa, but as it consists of a single layer of large rectangular protein rich cells covering many rows of starch filled cells increasing in diameter 5–30μ it is easily distinguishable. A small amount of endosperm residue may also be observed at times.

The typical black colour of the testa is given by a blue water soluble substance contained in the lower unsuberised portions of the Malpighian layer, and distributed especially in the cell contents. This colour is an anthocyan, as it turns red with glacial acetic acid, and is comparable to that in the coloured spots of certain seeds of *Melilotus alba*. It is sensitive to pH change, going red with acid, and blue to brown with alkali. The phloroglucin test for lignin therefore gives a positive reaction in the cell walls in which this pigment occurs, not confirmed in seeds with colourless coats. Contact with zinc causes the testa to change to green. During absorption of water in a soft seed, the dye passes out in solution through cracks in the upper parts of the Malpighian cells and stains surrounding paper or water. The loss of some of the colouring matter in this way, combined with decrease in concentration of the remainder through the increased area of the testa, causes the change from the black of a dry seed to the pink of a swollen one. A hard seed will give no stain if wet, since there is no way the dye can escape.

Investigation of the effect of wetting on the testa layers showed that the collapsed " nutrient " layer expands up to about three times its normal thickness, and the Malpighian cells by about 10%. Both layers expand this latter amount in width. The great increase in size of the nutrient layer through imbibition of water is a reversible process. This may mean that temporary wetting of soft seeds in the field may cause little damage.

The effect of water on a whole soft seed is to cause swelling in a similar way to that in a free section but the collapsed layer may not expand in depth so much. The swelling and consequent increase in volume of the testa causes a gap (demonstrable on cutting sections of freshly swollen seed). to form between it and the cotyledons within. These in turn absorb water and expand about 30% in diameter when they again fit closely to the testa till after germination.

The testas of both hard and soft seeds show the same physical and chemical organisation. Their functional difference is solely due to permeability in various localities of the soft seed. The seed that has dried out but has not become hard is permeable in one or more irregular patches over the seed coat, generally at or near the hilum, and is termed "original" soft seed. This permeability is due to incomplete formation of the suberinogenitic layer in the cell caps of those areas, as is evidenced by the absence of the light line. Such seeds may become hard by further drying out, which induces continuity of the impermeable layer.

Hard seeds become soft as a rule in one special locality, the strophiole, which is never permeable in original soft seed. This area is located on the long end of the seed near the hilum (fig. 10) and clefts through the light line will occur after sufficient local stress, e.g., fluctuating temperatures or bouncing. Present investigations on this clover, and local samples of lucerne, strawberry clover, white and sweet clover, confirm Hamly's discovery of the structural importance of the strophiole in the softening of hard seeds. Sections show that the Malpighian cells are bent in this area, not vertical as in the rest of the testa, and are 3–4 times as long. Table 13 and fig. 11 *a b*. It was seen previously that they develop more rapidly, even forming a light line before the seed dries out. This precocity is evidently linked with increased tension between the cells with consequent greater sensitivity to splitting

TABLE 13.
Size of Testa Cells.

—	Strophiole Cells.	Ordinary Testa Cells.
1. Depth cuticle to light line	18 μ	6 μ
2. Depth cuticle base Malpighian cells	140 μ	50 μ
3. Depth nutritional layer	20 μ	10 μ
4. Width Malpighian cells	6 μ	8–10 μ

Fig. 11

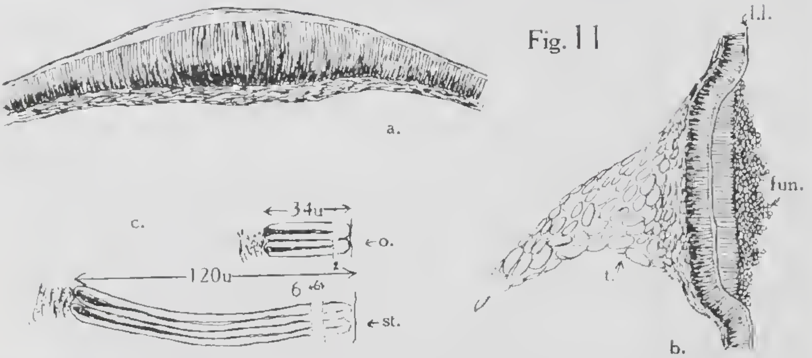


FIG. 11.—Longitudinal sections (*a*) through the strophiole of a hard seed, showing the increased size of the Malpighian cells, and their bent positions; (*b*) through the hilum, showing the continuity of the lightline, and the loose cells of the funicle, left after separation of the seed on drying; (*c*) comparison of Malpighian cells at strophiole and elsewhere in the testa. (fun. = funicular cells, l.l. = lightline, t. = tissue between radicle and cotyledons, st. = strophiole, o. = ordinary testa cells.)

than elsewhere. In Plate XII., F, the penetration of osmic acid at the strophiole of a softened seed is evident, and contrasts with that in a seed before drying (Plate XII., E).

Discussion.

The significance of hard seeds in the soil is important in all clovers and medicks, and is of special interest with reference to subterranean clover as a component of the pastures of southern Australia since this plant is an annual, depending for its persistence and spread on establishment from seed.

The plant dies at the onset of dry conditions in early summer, and if there has been successful burr production, the matured seed is capable of germination from December onwards, but only a small percentage is "soft." Heavy rain is sufficient to germinate this. But the dry weather that usually follows soon kills all or most of the seedlings. The same will occur after any further "breaks" in the hot dry conditions until with the autumn rains, a level of available moisture is reached which is adequate for establishment of seedlings. Observations show that normally there is no further germination till spring when a further burst of seedlings appears. Such irregular germinations will continue in the next years till all have softened. In some years, the first heavy rains occur in autumn, and therefore are followed by very heavy germination, and the hard seeds left, later give rise to seedlings for which there are no openings in the sward. If there are no heavy rains till winter, seedling establishment will be so poor owing to low temperatures, that further spring germination will be very useful in filling spaces in the "open" pastures.

Under some circumstances, the presence of hard seeds is an insurance, under others superfluous in the field, in others when the total number of soft seeds is small, is a definite deterrent to a quick even establishment of a thick stand.

Burial of the burr in subterranean clover has been regarded as a means of securing a larger number of soft seeds, and hence heavier seedling establishment. Present investigations have indicated that in well developed seeds, dried in the burr at the same time, those dried above ground will form a greater percentage of hard seeds, because of the higher saturation deficit; usually however, the buried seeds develop further because of a moister environment, and their later drying results in higher hardseededness, which is also generally of longer duration.

The fact of hardseededness seems to depend on a continuity of an impermeable suberinogenitic thickening with consequent evidence of a lightline, on the top of the Malpighian cells. This continuity is dependent on (1) the tendency of the cells to deposit the thickening according to an inherited capacity; (2) time for deposition; (3) degree of dehydration.

Workers with other legumes such as lupins and sweetclover. have shown the practicability of selecting more permeable seeded lines. In subterranean clover, the individual variation in hard-seededness indicates the possibility of selection here also. Stevenson (9) indicates that the more permeable strains in *Melilotus alba* are so because of lack of continuity of the suberin round the testa.

The quality of hardseededness depends on the tendency of the strophiolar cells to split. The evidence is that long development of the seed before drying out, and high degree of drying results in hard seed of long duration. It is probable from this that this splitting is a function of (1) tension between cells; (2) thickness of suberin at shoulders of cell caps; (3) degree of dehydration.

Variations in this quality are the cause for the gradual softening through the years. The variability in rapidity of splitting at the strophiole under stress seems to be related more to conditions of ripening than to strain differences, and may be influenced by varying the time of flowering and ripening.

Preliminary observations have shown that reliable assessment of the establishment value of hard seeds of this clover, in Victoria, according to their germination by mid-April, and by September the next spring would be of use in the field.

This knowledge is of no importance in good quality commercial seed because of the efficiency in softening, of machine harvesting methods.

In fig. 12 the present knowledge on maturation and germinability of the seed of *T. subterraneum* is summarized. It corresponds closely to that of *Cajanus indica*, Dutt (3).

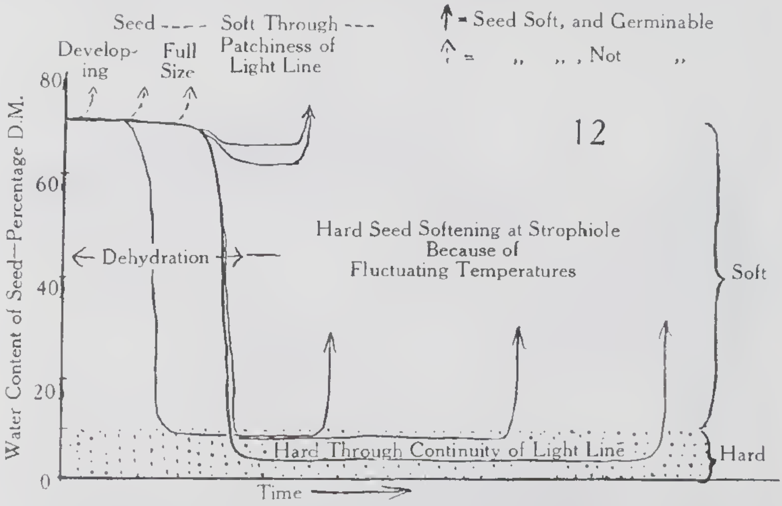


Fig. 12.—Summary of the behaviour of the seed of *Trifolium subterraneum* after growth to maximum size, indicating the relation of softness to germinability, and that of hardness to degree of dehydration, and to continuity of the lightline.

Further work may develop methods of controlling the process of seed development in a desired stage of maturity, e.g., a large percentage of original soft seeds, or of seeds capable of remaining hard for short or long periods.

Summary.

A full sized seed (not yet dried and fully mature) in the burr is always permeable over part of its surface, due to discontinuous development of the impermeable layer.

The drying out of these seeds results in some being sufficiently dried to develop a continuous layer of suberinogenitic character, and these form the "original" hard seeds in the burrs. Others, less dehydrated, remain permeable over part of the surface and form the "original" soft seeds. These may become hard on further drying.

Development of hard seeds is dependent on continuity of the suberin layer over the distal ends (caps) of the Malpighian cells of the testa. This continuity depends on degree of dehydration and thickness of deposition, which in turn are based on conditions of ripening in the burr.

The quality of the hardness, e.g., softening within a few weeks in the soil (pseudo-hardness), as compared with hardness lasting up to a year or more is controlled by the tension of the strophiole cell walls and the toughness of the suberin. These depend on rate of seed development, and degree of drying.

Hard seeds become soft through sensitivity of the strophiole cells to splitting under pressure. There is no reversion to hard on further drying, since a split between cells through the light line, once developed, is not sealed.

Softening of a hard seed through a split in the strophiole results from widely alternating temperatures or from freezing and thawing in the soil, and from the "bouncing" action in cleaning machinery.

Soft seeds from the burr differ from hard either in the presence of a split between cells at the strophiole, or in permeable patches in the rest of the testa. Those from commercial seed samples may also be caused by surface scratching.

Hard seeds are present in Victorian samples of subterranean clover burr to the extent of 55–90 per cent.; and in the commercial samples from 4–50 per cent. according to the roughness of the huller.

The percentage and quality of hard seeds produced by a plant of subterranean clover is controlled by seed development and degree of drying. The importance to these factors of time of flowering, panicle development, seed development above or below ground, time of burr drying, death of plant, time of harvesting, and individual plant variation within a strain, has been shown. It remains to get further data on the extent of softening in the first autumn, and to investigate the effect of strain apart from

time of flowering, and of selection within a strain, on the percentage and quality of hard seeds, before the practical value of field variation can be assessed.

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Plates.

Plate XI.

(A) A small plant of subterranean clover, showing prostrate habit, and formation of burrs along the runners.

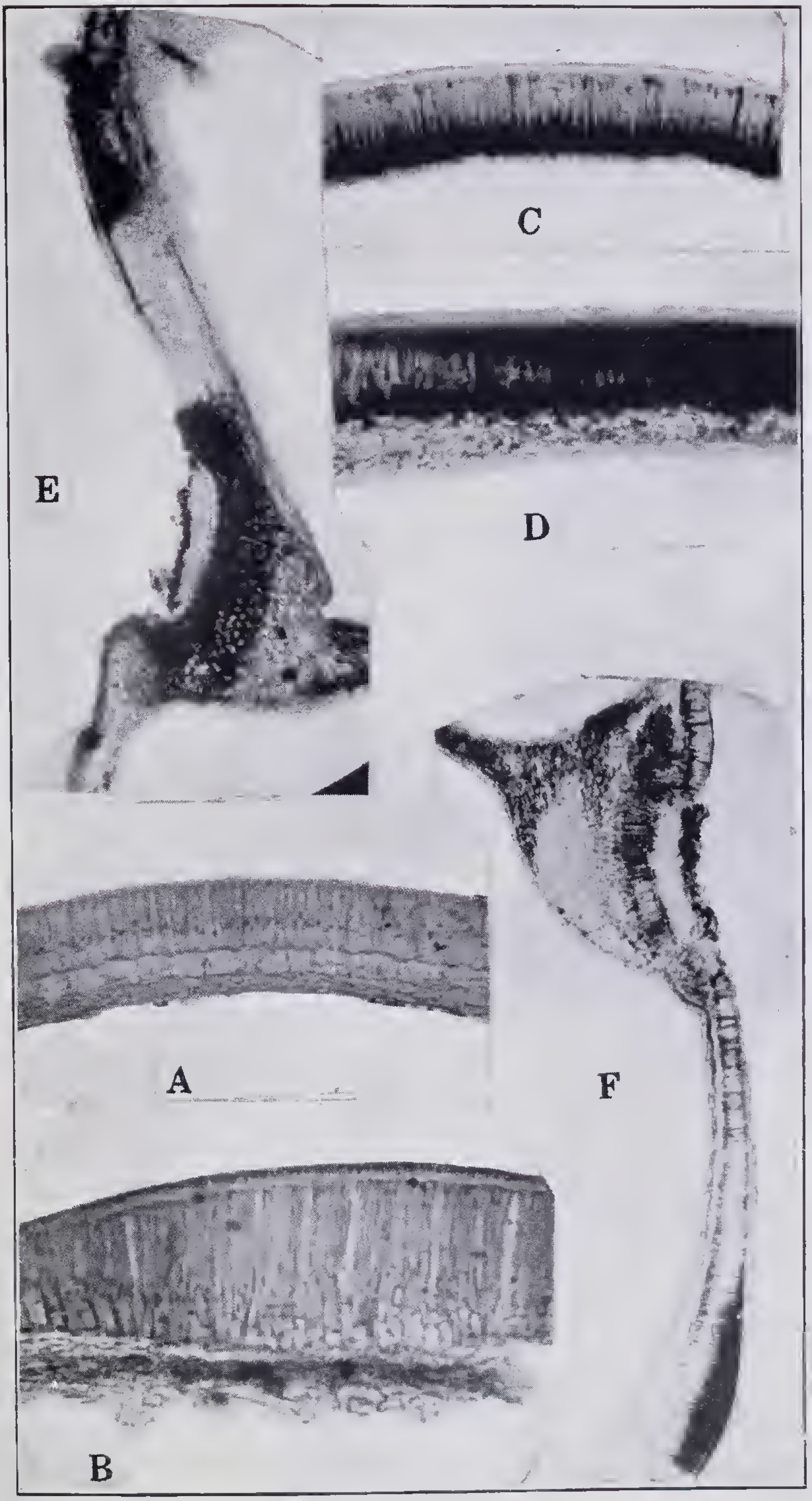
(B) Portion of a well developed runner, showing the stages from erect panicle to full grown burr.

(C) (i) Burr developed below the surface; (ii) Various views of burrs developed above ground; (iii) Pods from one burr; (iv) Full grown seeds; (v) Dried seeds.

Plate XII.

Sections: (A). through testa of half grown seed, showing nucleate, unthickened, Malpighian cells, × 250; (B), through strophiole of same, showing a conspicuous lightline, and much longer Malpighian cells, × 250; (C), through testa of a hard seed, showing development of colour (especially in the cell lumens), lightline, thickening of cells, and absence of distinct nuclei, × 250; (D), the same, with the thickness of matrix defined by the spread of osmic acid staining below the lightline from a nearby scratch, × 350; through hilum and strophiole of seeds after treatment with osmic acid; (E), in a fully developed seed, the black stain shows entry of acid round hilum, near strophiole and elsewhere, × 80; (F), in a hard seed turned soft, entry is at the strophiole only, × 80.

Aitken—Subterranean Clover.



Aitken—Subterranean Clover.